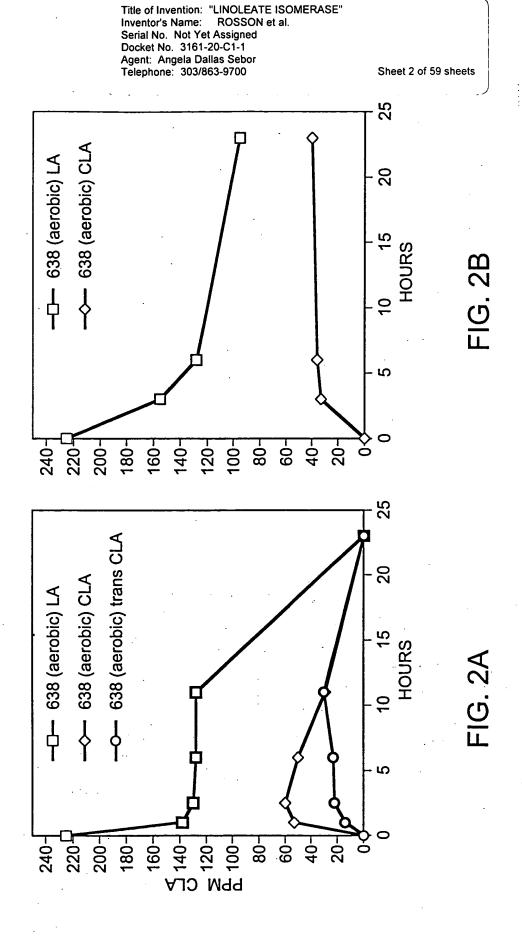
Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Sheet 1 of 59 sheets Telephone: 303/863-9700 25762 (anaerobic) CLA -□- 25762 (anaerobic) LA S 80-60-40-20-100-160-140-200-180-25762 (aerobic) trans CLA 25762 (aerobic) CLA —П— 25762 (aerobic) LA 5 80-60-40-20-PPM CLA 180구

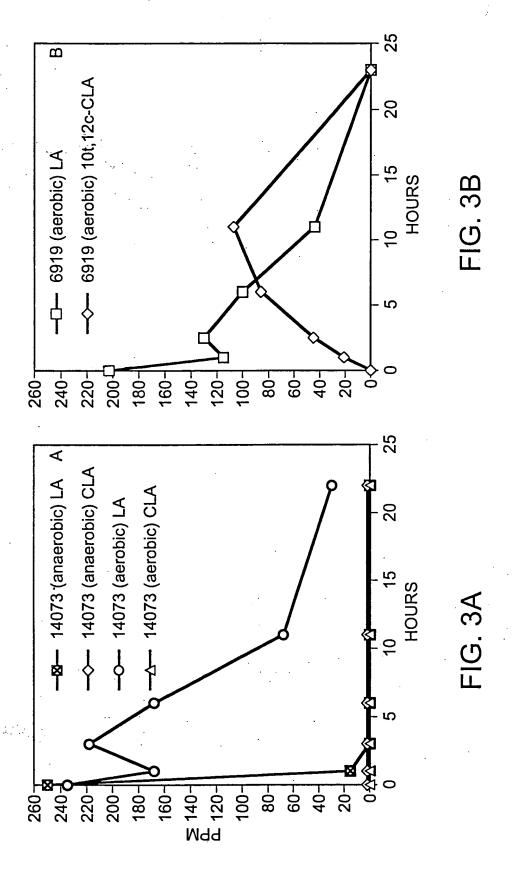
Title of Invention: "LINOLEATE ISOMERASE" Inventor's Name: ROSSON et al.

Serial No. Not Yet Assigned



Inventor's Name: ROSSON et al. Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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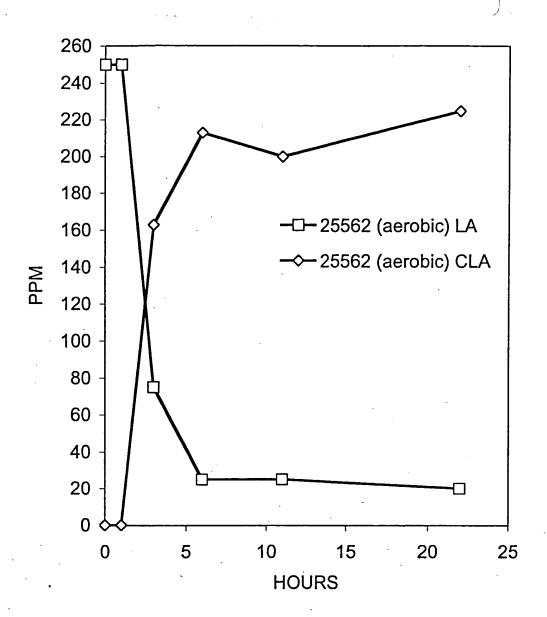


FIG. 4

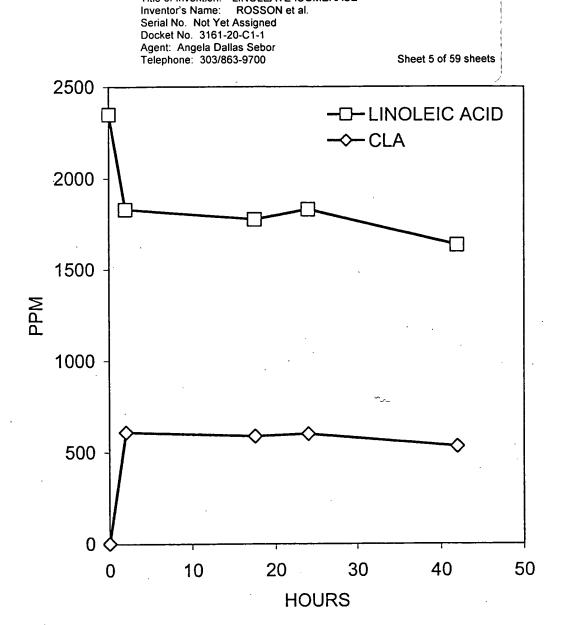
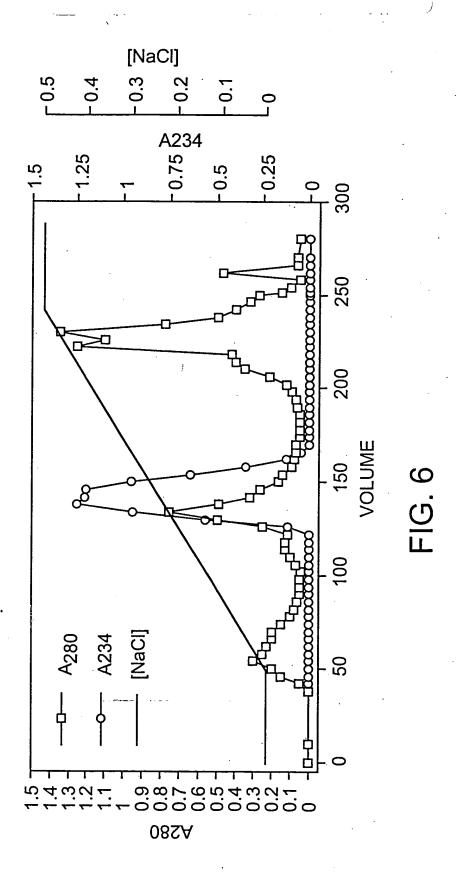


FIG. 5

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Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

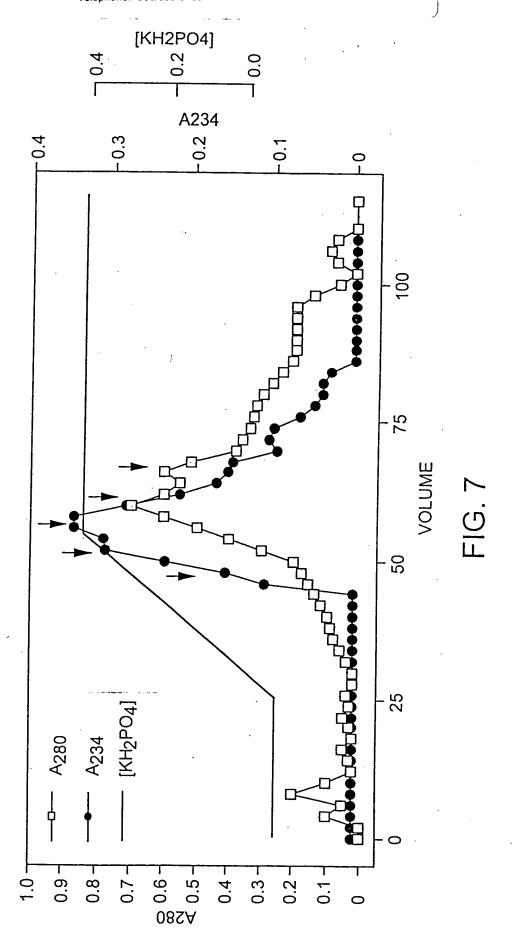
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Docket No. 3161-20-C1-1

Agent: Angela Dallas Sebor Telephone: 303/863-9700

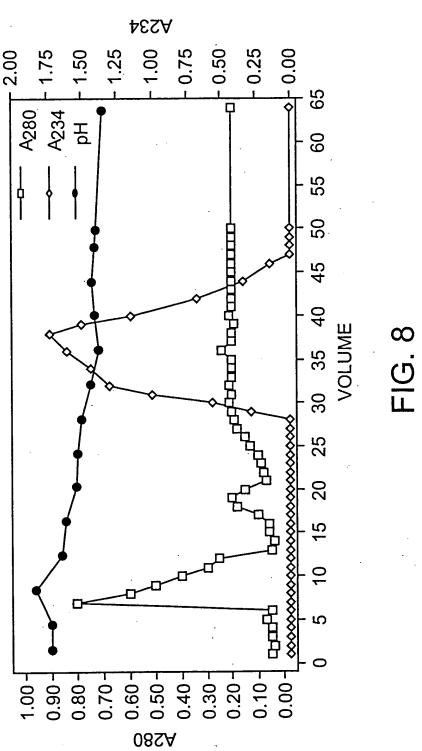
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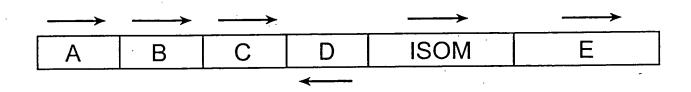


FIG. 9

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Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

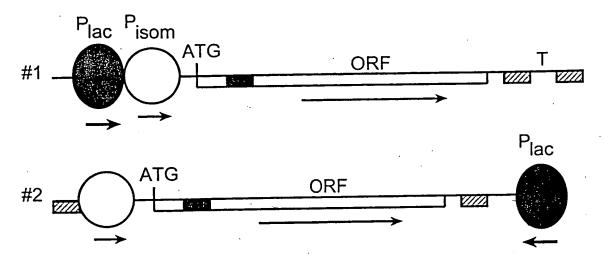
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FIG. 10

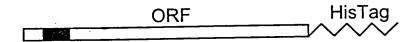
Inventor's Name: ROSSON et al. Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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## **Tested Two Constructs**



## New Construct #3:



## New Construct #4:



FIG. 11

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#### **Expression System:**

HaplI promoter

LAT promoter

- with the secretion signal peptide
- --- without the secretion signal peptide

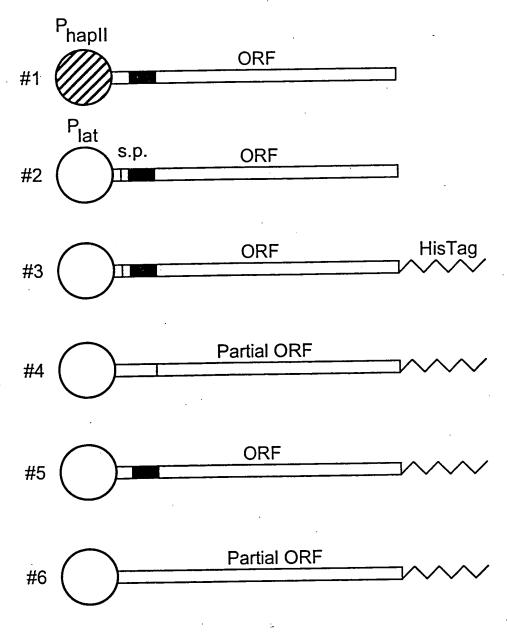


FIG.12

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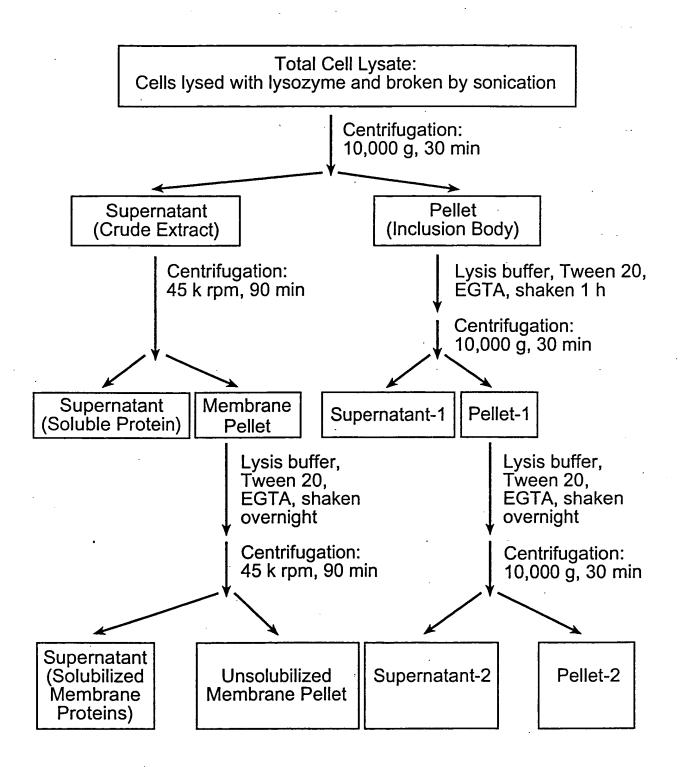


FIG. 13

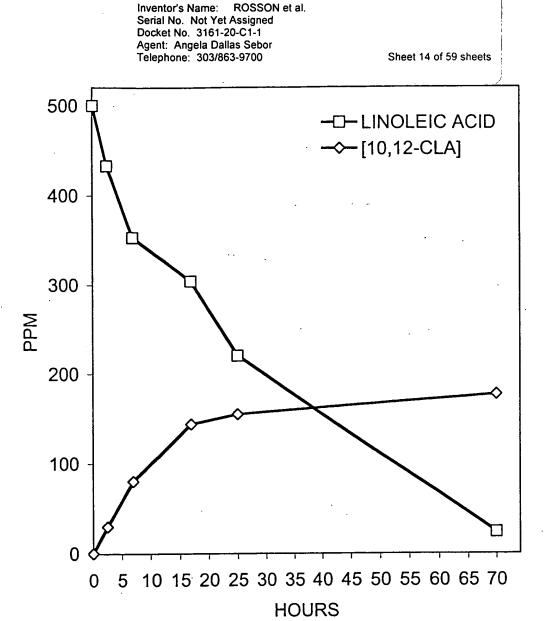


FIG. 14

Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Sheet 15 of 59 sheets Telephone: 303/863-9700 P. acnes Culture Harvest, wash, Suspend in Buffer French Press 15K/30 minutes **Crude Extract** (A) Soluble (B) 45K/90 minutes Particulate Fraction Fraction Buffer Wash Washed (C) 45K/90 minutes Pellet

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**FIG.15** 

45K Wash

(D)

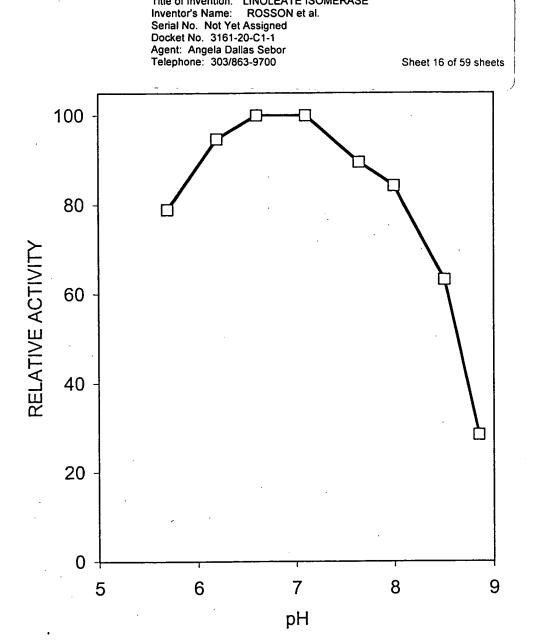


FIG. 16

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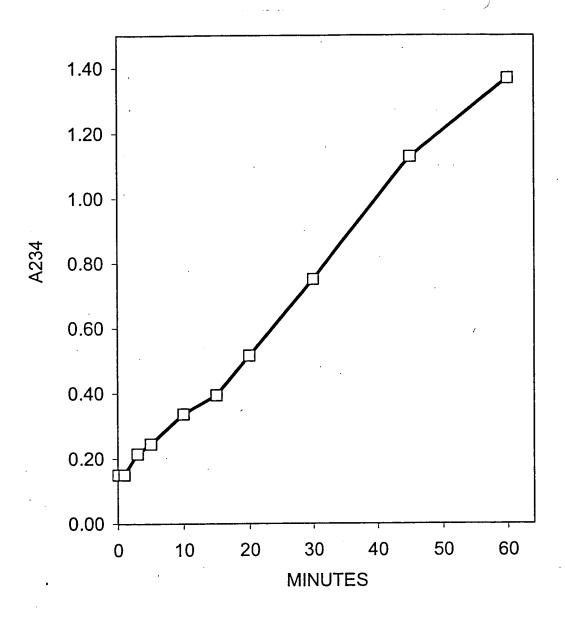


FIG. 17

Inventor's Name: ROSSON et al. Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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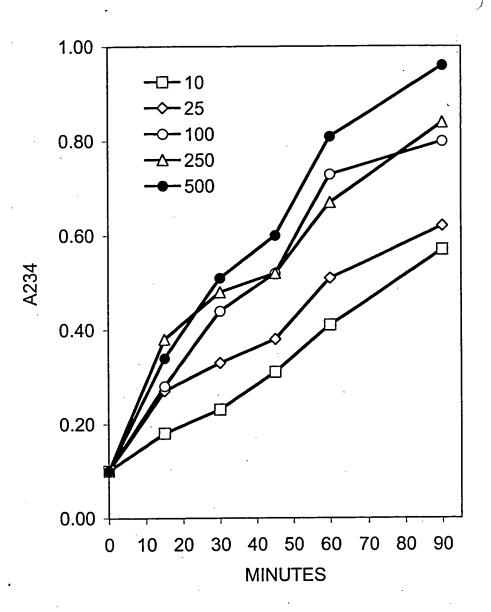


FIG. 18

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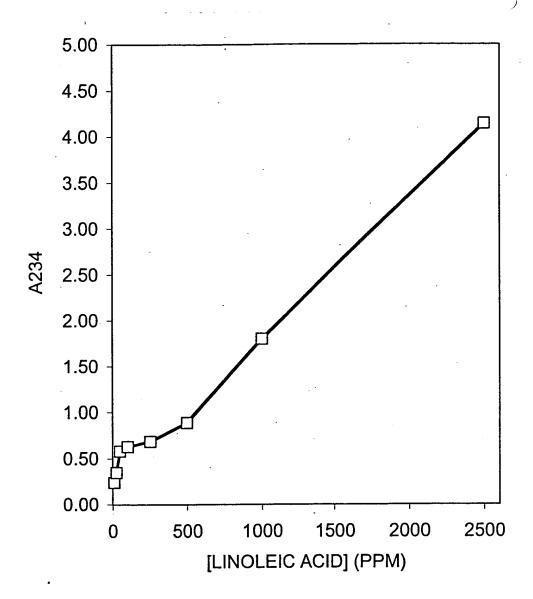
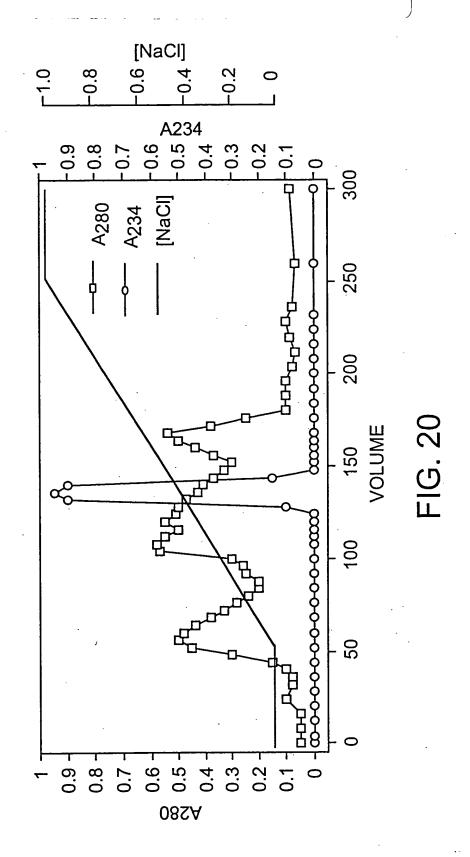


FIG. 19

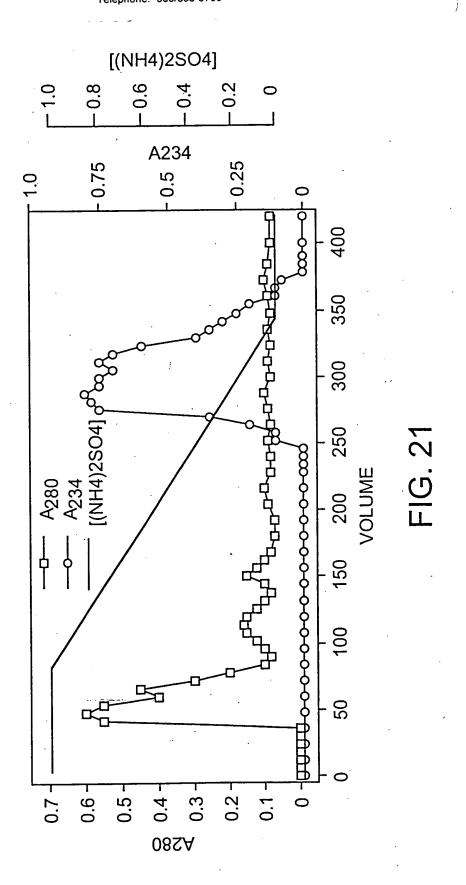
Inventor's Name: ROSSON et al. Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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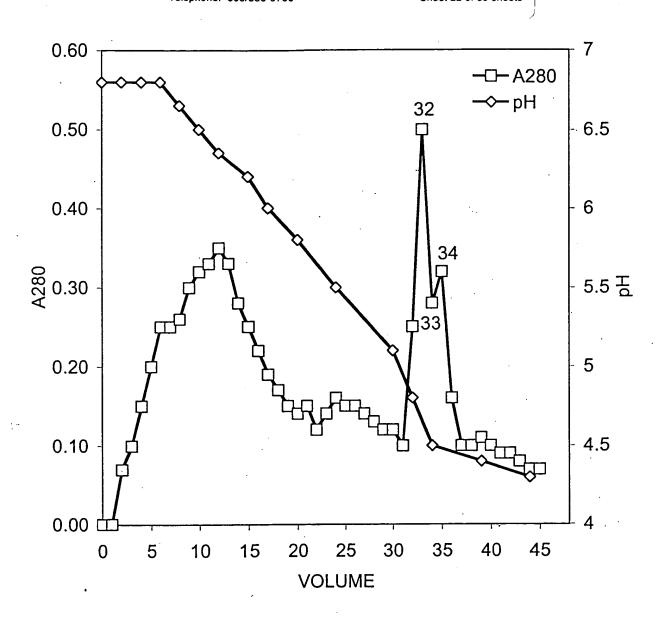
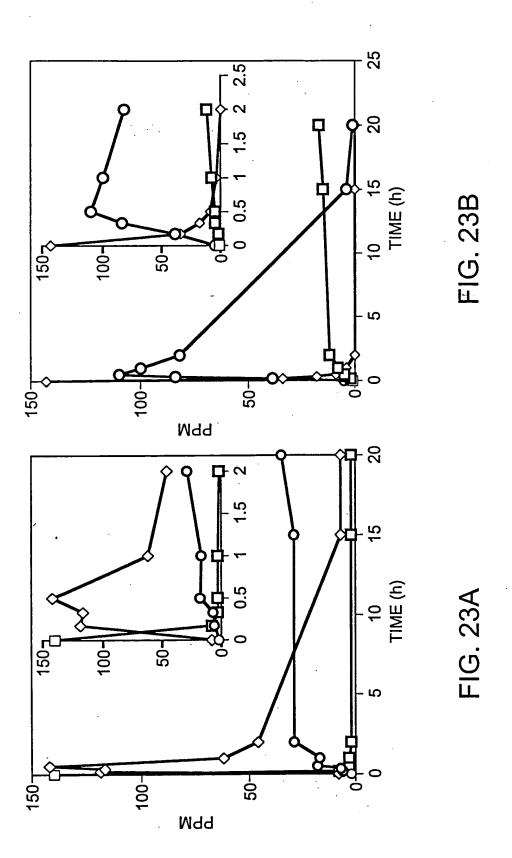


FIG. 22

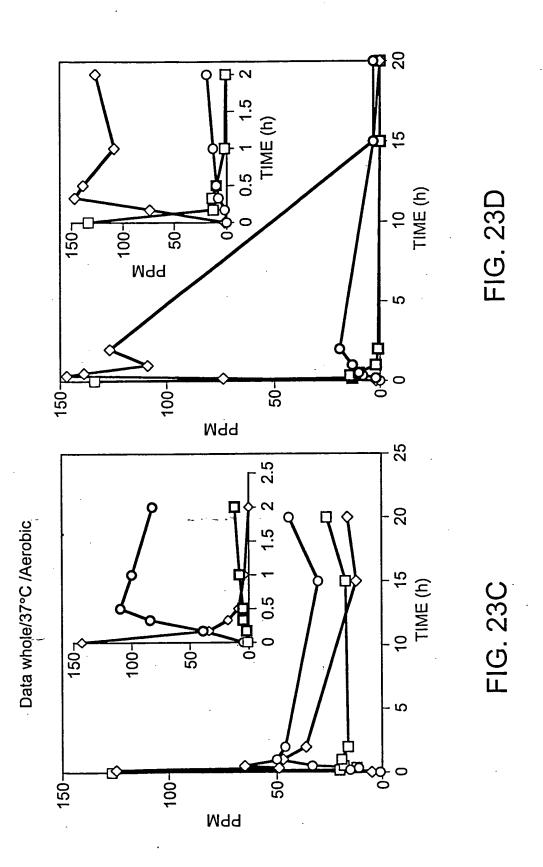
Title of Invention: "LINOLEATE ISOMERASE" Inventor's Name: ROSSON et al. Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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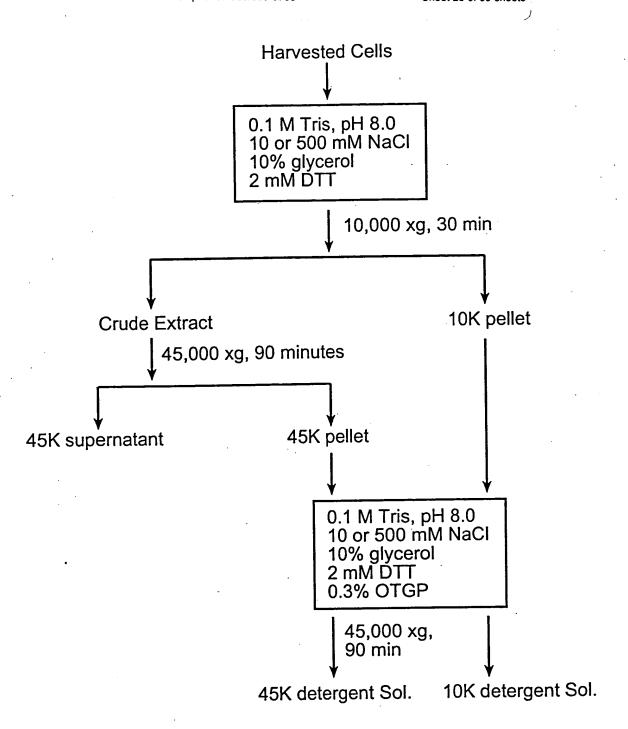


FIG. 24

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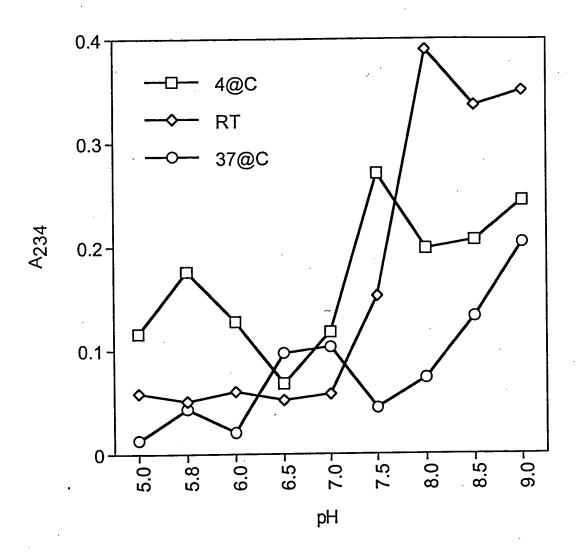
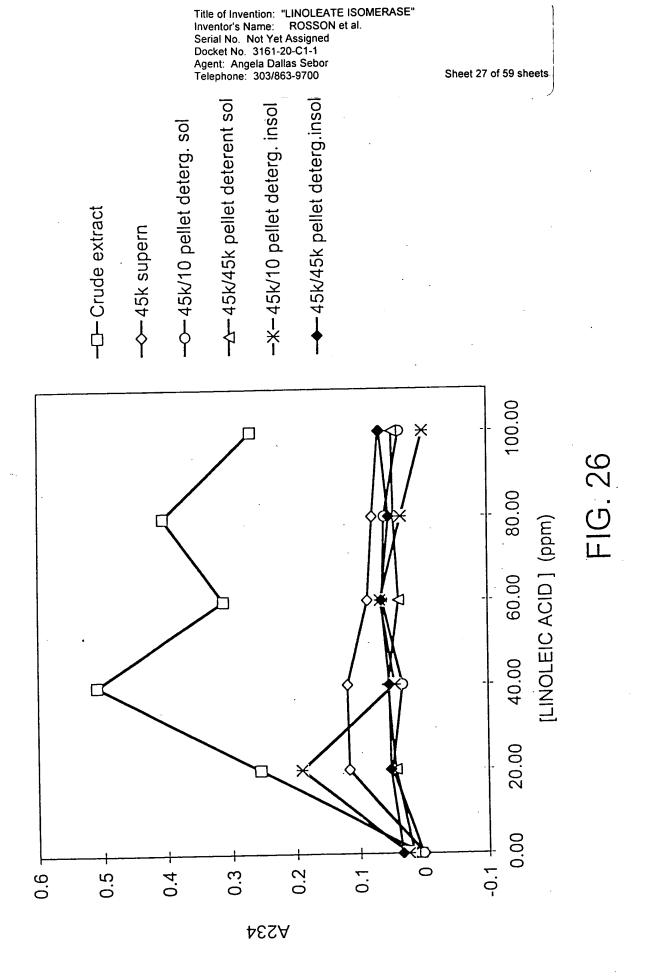


FIG. 25



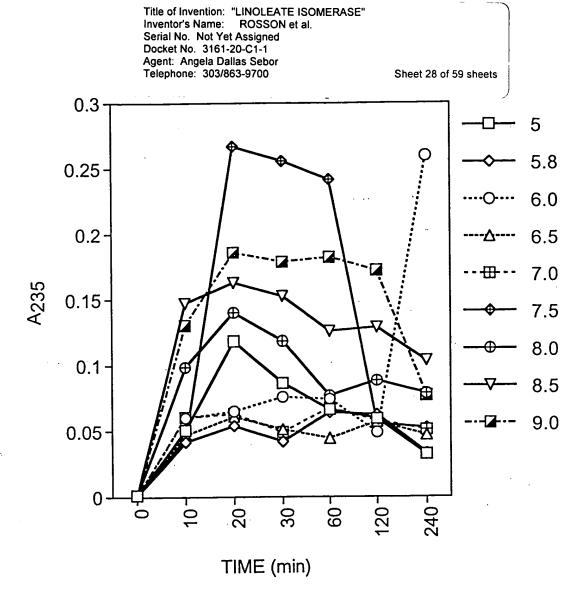


FIG. 27

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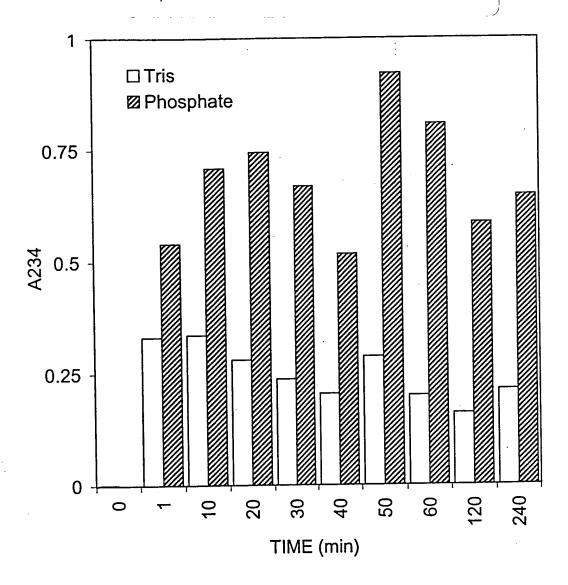
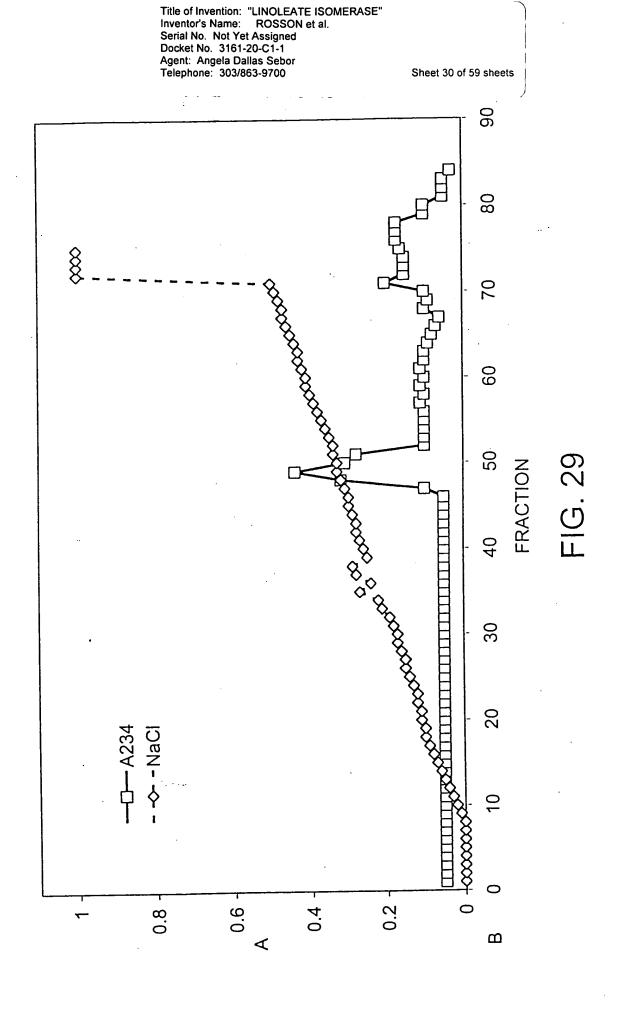


FIG. 28



Title of Invention: "LINOLEATE ISOMERASE" Inventor's Name: ROSSON et al. Serial No. Not Yet Assigned Docket No. 3161-20-C1-1

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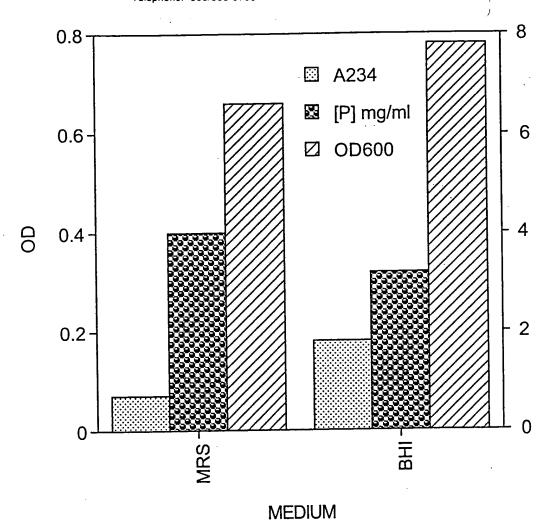


FIG. 30

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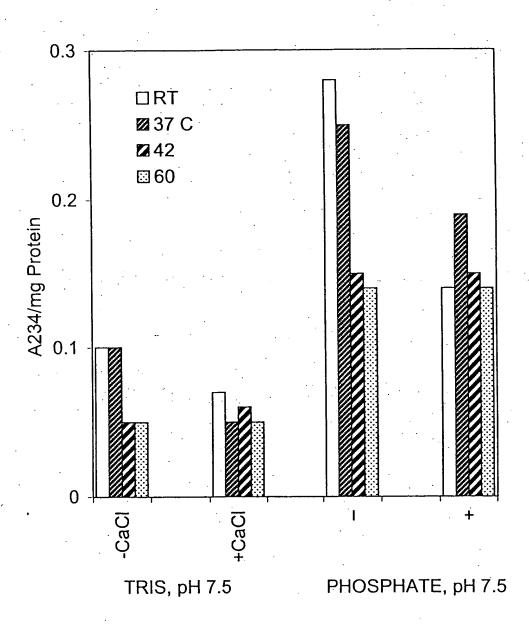


FIG. 31

Title of Invention: "LINOLEATE ISOMERASE" ROSSON et al.

Inventor's Name: ROSSON Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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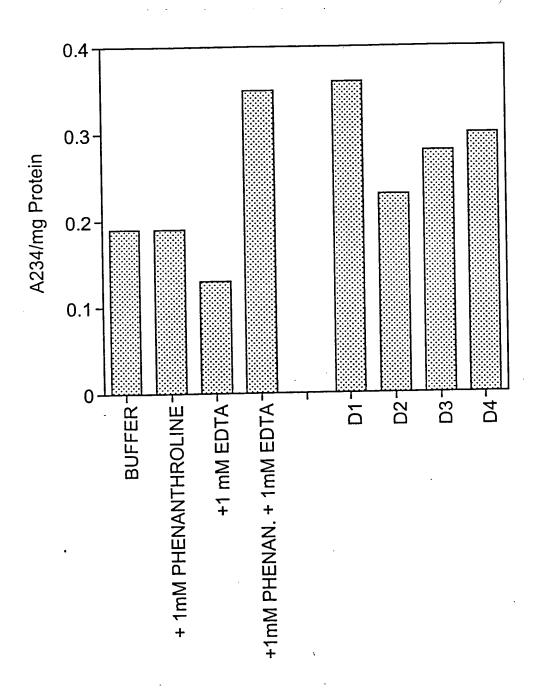


FIG. 32

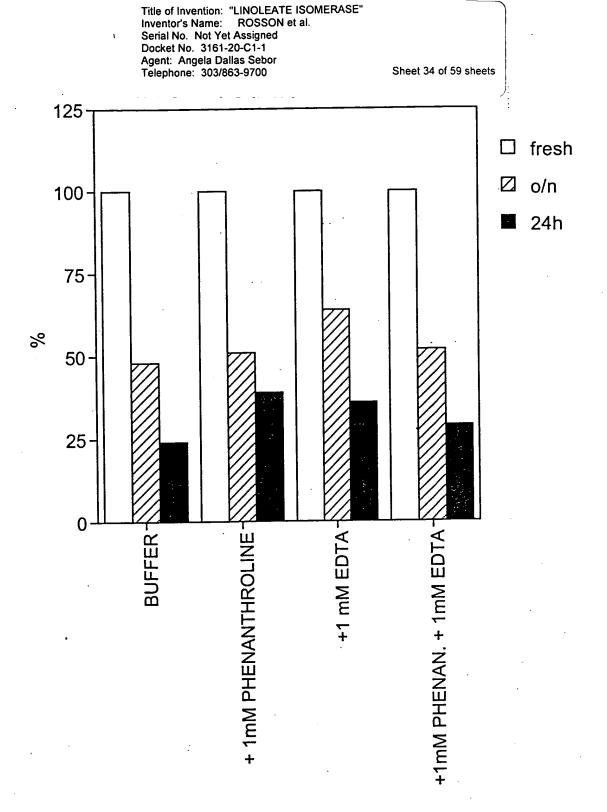


FIG. 33

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# pH Effect on Extraction Efficiency of Isomerase

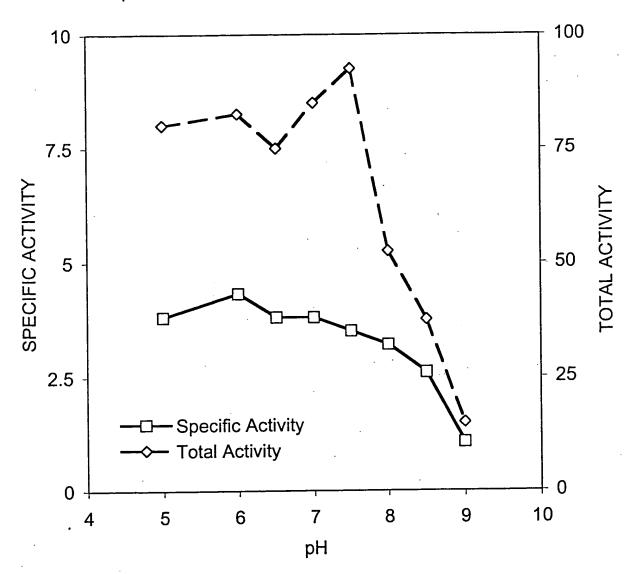


FIG. 34

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### Half Lives of Isomerase vs pH

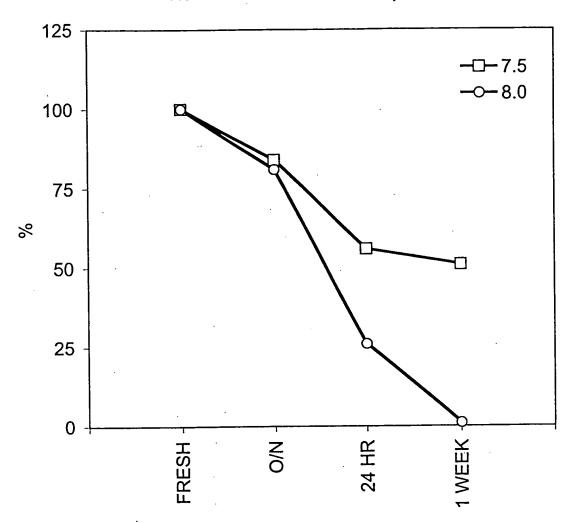
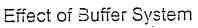


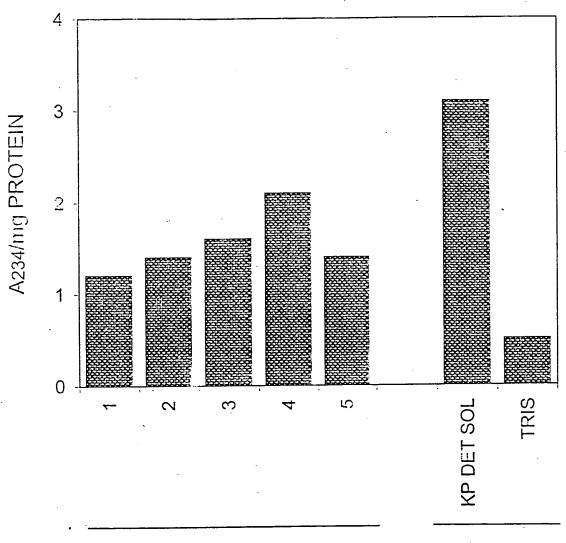
FIG. 35

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CRUDE EXTRACT

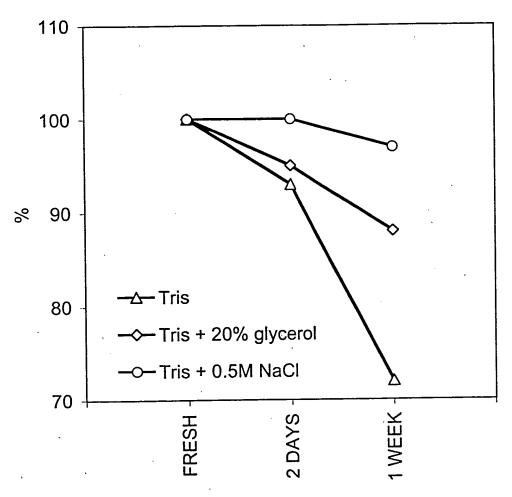
DETERGENT SOL. FRACTION

FIG. 36

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# Effect of Glycerol and Salt Concentration on Isomerase Stability



AGE OF ISOMERASE

FIG. 37

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### Stability of Detergent Solubilized Isomerase

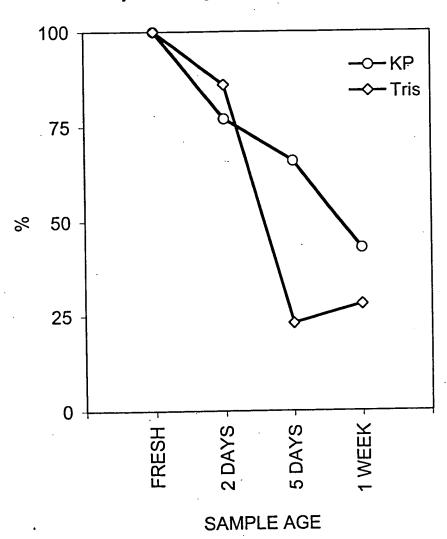


FIG. 38

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### Chromatography on Mono Q Column

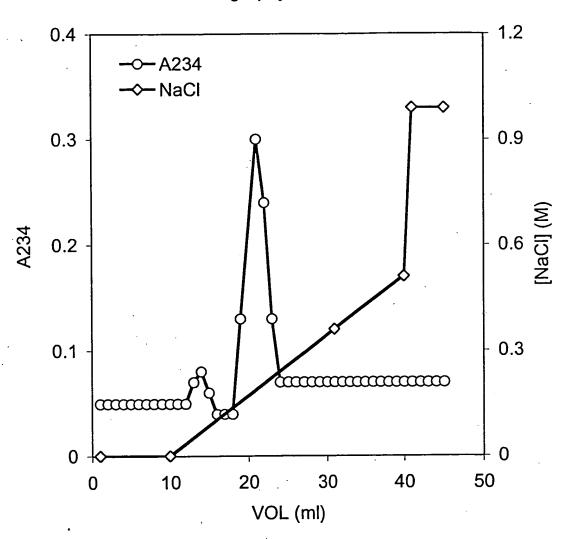


FIG. 39

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# Separation of Partially Purified Isomerase by Chromatofocusing

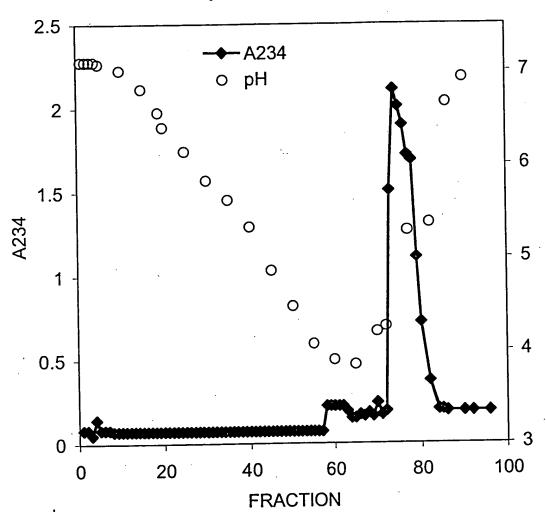
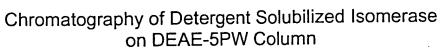


FIG. 40

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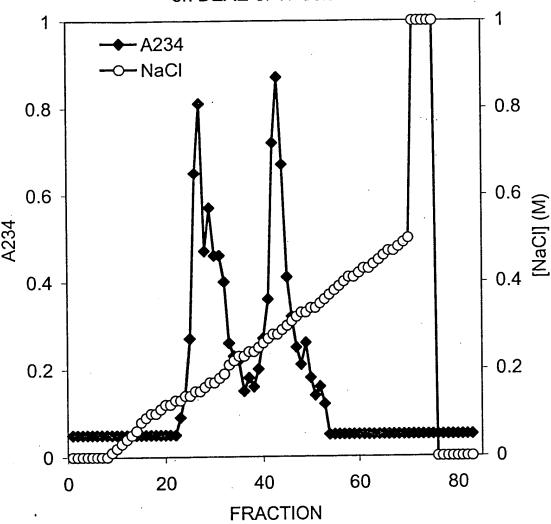


FIG. 41

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M 1 2 3 4 5 6 7 8 9

KDa

70
55
45

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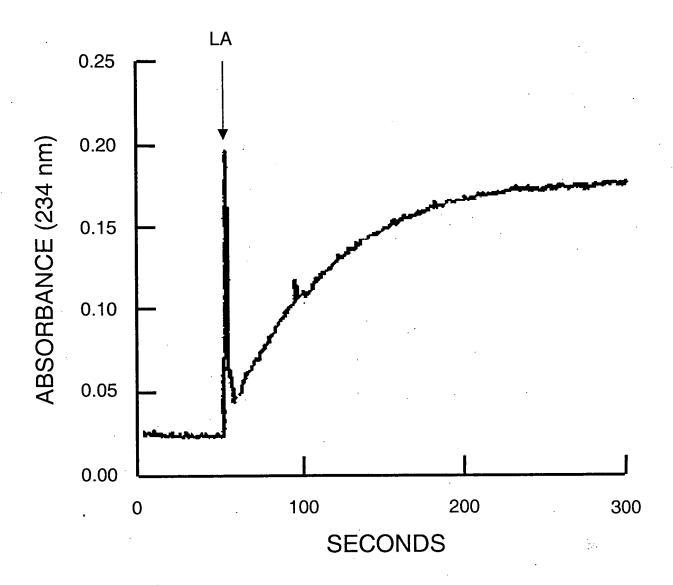
Western Blot analysis of linoleate isomerase using rabbit antibodies specific for the cloned *L. reuteri* PYR8 isomerase. Total protein of cell lysates prepared from different strains was used in the analysis.

- M. Protein size marker
- 1. E. coli expressing the isomerase-histag fusion protein
- 2. L. reuteri PYR8
- 3. B. subtilis wild type
- 4. *B. subtilis* transformed with the vector pBH1 containing the isomerase gene under HpaII promoter control
- 5. L. reuteri 23272 wild type
- 6. *L. reuteri* 23272 transformed with the vector pTRKH2 containing the isomerase gene under the control of both its native promoter and the *lac* promoter
- 7. *L. reuteri* 23272 transformed with the vector pTRKH2 containing the isomerase gene under the control of its native promoter
- 8. P. acnes 6919
- 9. C. sporogenes 23272

FIG. 42

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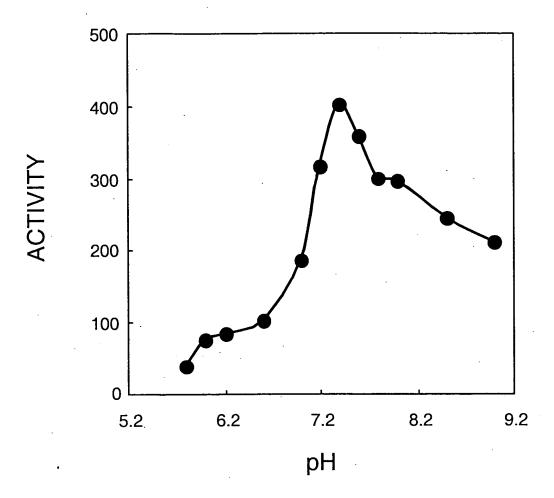
Time course of isomerization of linoleic acid. The isomerization reaction was initiated by adding 20  $\mu M$  linoleic acid at 50 seconds.

FIG. 43

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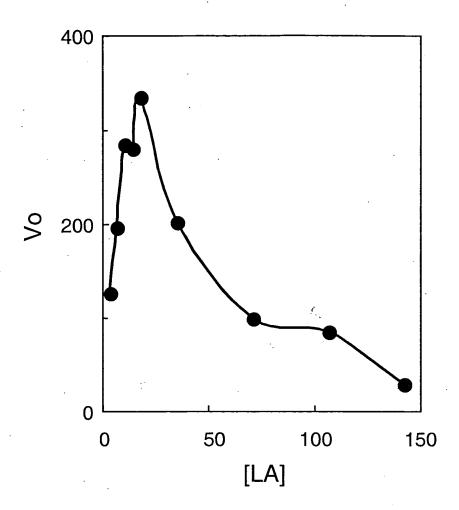


Effect of pH on isomerization of linoleic acid to CLA by *C. sporogenes* linoleate isomerase. Activity as nmol CLA/min/mg protein.

FIG. 44

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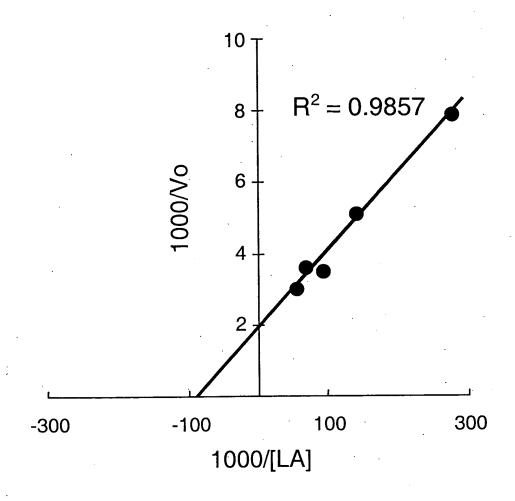


Effect of linoleic acid concentration on the rate of isomerization. Vo as nmols CLA/min/mg protein. [LA] as  $\mu M$ .

FIG. 45

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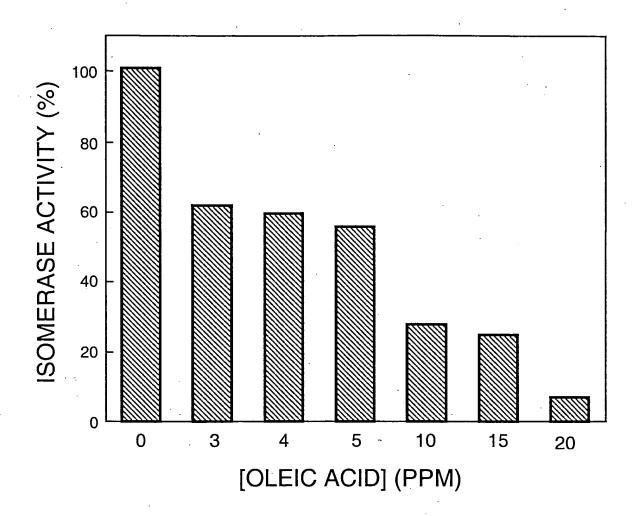


Lineweaver-Burk plot of reaction kinetics of  $\it C. sporogenes$  linoleate isomerase. Vo as nmols CLA/min/mg protein. [LA] as  $\mu M$ .

FIG. 46

Inventor's Name: ROSSON et al. Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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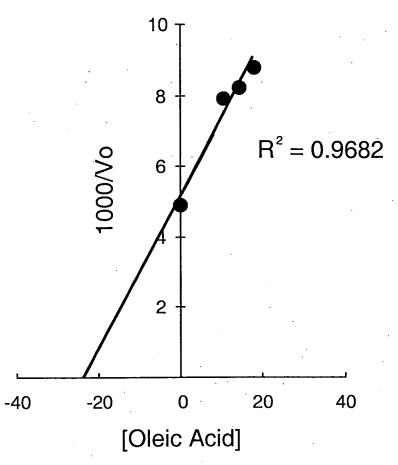
Effect of oleic acid on isomerase activity with linoleic acid as substrate. The concentration of linoleic acid was fixed at 36  $\mu$ M<sub> $\odot$ </sub>. Oleic acid was added at the indicated concentrations.

FIG. 47

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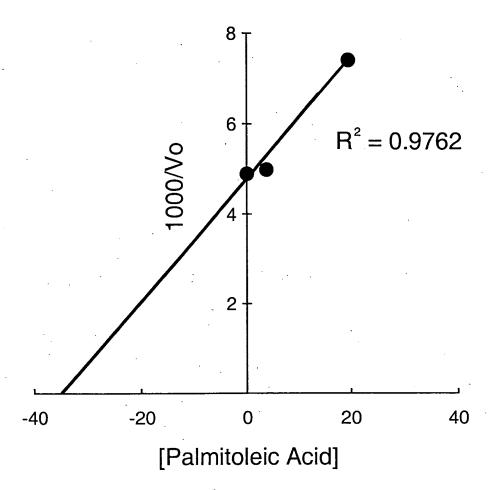


Secondary plot of oleic acid inhibition. Vo as  $\mbox{ nmols }$  CLA/min/mg protein. [Oleic Acid] as  $\mbox{ }\mu\mbox{M}$ 

FIG. 48

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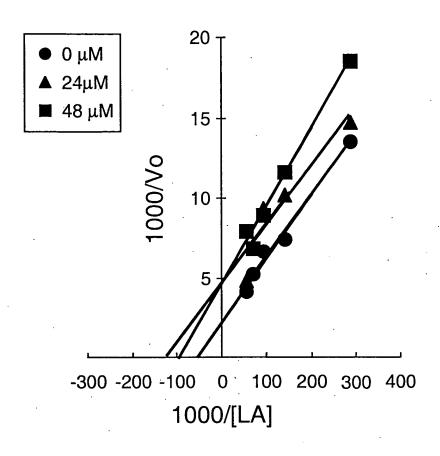
Secondary plot of palmitoleic acid inhibition. Vo as  $\mbox{nmols}$  CLA/min/mg protein. [Palmitoleic Acid] as  $\mbox{\mu}M$ 

FIG. 49

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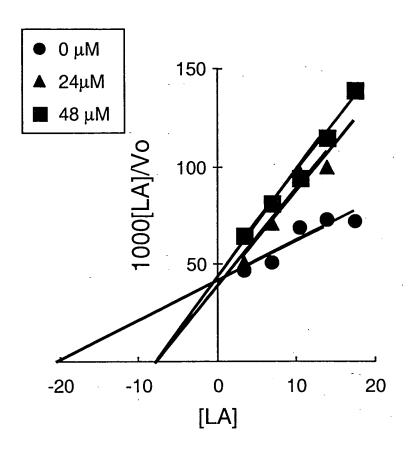


Linweaver-Burk plot of linoleic acid isomerization kinetics in the presence or absence of oleic acid. Vo as  $nmols\ CLA/min/mg\ protein.\ [LA]\ as\ \mu M.$ 

FIG. 50

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Hanes-Woolf plot of oleic acid inhibition of linoleic acid isomerization kinetics. Vo as nmols CLA/min/mg protein. [LA] as  $\mu M$ .

FIG. 51

Title of Invention: "LINOLEATE ISOMERASE"
Inventor's Name: ROSSON et al.
Serial No. Not Yet Assigned
Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

Sheet 53 of 59 sheets

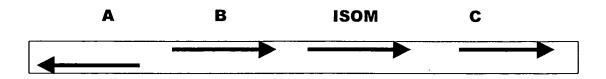


FIG. 52

Title of Invention: "LINOLEATE ISOMERASE" ROSSON et al.

Inventor's Name: ROSSON Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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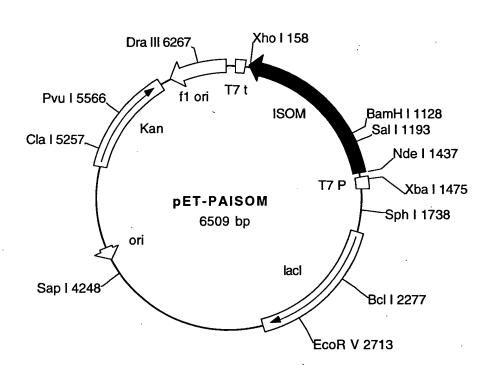


FIG. 53

Title of Invention: "LINOLEATE ISOMERASE" ROSSON et al.

Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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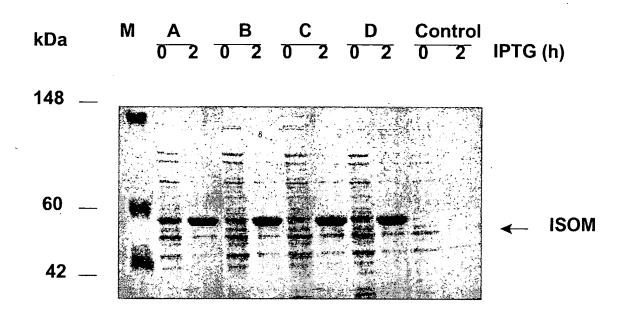


FIG. 54

Title of Invention: "LINOLEATE ISOMERASE" Inventor's Name: ROSSON et al. Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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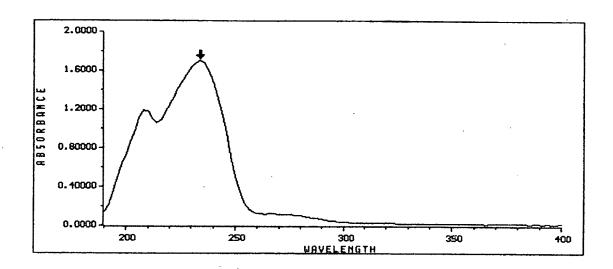


FIG. 55

Title of Invention: "LINOLEATE ISOMERASE" Inventor's Name: ROSSON et al.

Serial No. Not Yet Assigned

Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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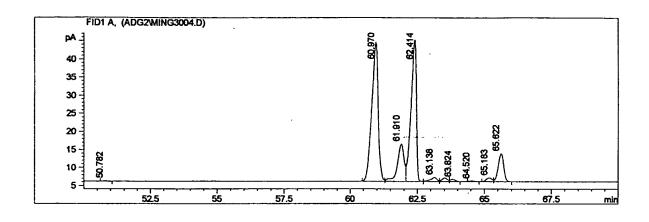


FIG. 56A

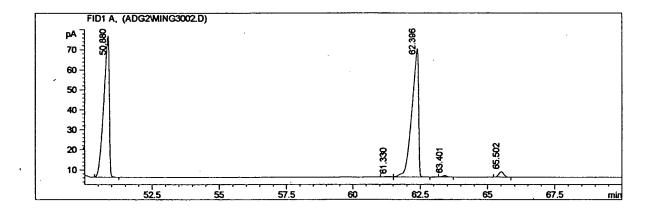
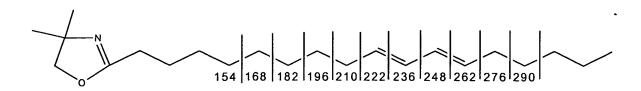


FIG. 56B

Inventor's Name: ROSSON et al.
Serial No. Not Yet Assigned
Docket No. 3161-20-C1-1
Agent: Angela Dallas Sebor
Telephone: 303/863-9700

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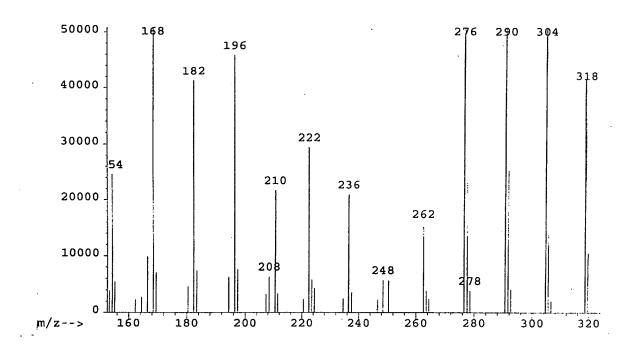


FIG. 57

Title of Invention: "LINOLEATE ISOMERASE" Inventor's Name: ROSSON et al. Serial No. Not Yet Assigned Docket No. 3161-20-C1-1 Agent: Angela Dallas Sebor Telephone: 303/863-9700

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# A putative NAD-binding domain shared by linoleate isomerases and some other enzymes

# NAD-binding domain

(SEQ ID NO:73)	(SEQ ID NO:74)	(SEQ ID NO:75)	(SEQ ID NO:76)	(SEQ ID NO:77)	(SEQ ID NO:78)	(SEQ ID NO:79)	(SEQ ID NO:80)	(POS. 2-59 OF SEQ ID NO:61)	(POS. 19-79 OF SEQ ID NO:18)	
0BB	183 SEAYSAKIALF*A*PASISC*SF*ARL*YSDITIF*KQEYV**	KVAIV*A*LS*LVV*SE*LHA*IDDVTLY*ASDRI**KLWS	VKTGKKVAVV*S*PA*LAA*QQ*ARA*-HDVTVF*KNDRV**RIEQ	VV*G*FS*LKA*RD*TNA*-KKVLLL*GGERL**RAYS	RIAII*A*LA*MAT*VE*VDA*-HEVELY*ARSFI**KVGSWVDGD*NHI-EM*	STSKRPTAIVI*S*VG*VST*AR*ARA*FH-VTVL*KNNFT**RCSL-IHHE*YRF-DQ*	RVIVV*A*MS*ISA*KR*SEA*ITDLLIL*ATDHI**RMHK-TNFA*INV-EL*	: SISKDSRIAII*A*PA*LAAGMY*EQA*FHDYTIL*RTDHV**KCHS-PNYH*RRY-EM*	19 GVDKK-HAYIV*G*LA*LSA*VF*IRDAQMP*-ENIHIL*ELPVA**SLDG-EDRP*IGFVTR*	#1: Dihydropyrimidine dehydrogenase (Human), Q12882 #2: Tryptophane monoxygenase (Agrobacterium vitis), AAC77909.1 #3: Glutamate synthase (Deinococcus radiodurans), AAF09769.1 #4: 6-hydroxy-L-nicotine oxidase (Arthrobacter nicotinovorans), AJ223391 #5: \$\zeta_{\text{caroten}}\$ desaturase (Synechocystis sp.), D90914 #5: \$\zeta_{\text{caroten}}\$ desaturase (Cercospora nicotianae), P48537 #7: Polyamine oxidase (Zea mays), O64411 #8: (t, c)-10,12-Linoleate isomerase (Propionibacterium acnes) #9: (c, t)-9,11-Linoleate isomerase (Lactobacillus reuteri)
CONSENSUS	#1 183	#2 8	#3 140	#4 6	#5 8	#6 3	#7 8	#8 2	#9 19	SEQUENC #1: Dih #2: Try #3: Glu #4: 6-h #5:  #6: Phy #6: Phy #8: (t,